**REMARKS**

Applicants are submitting this amendment supplementally to their amendment of 17 December 2007 in order to reflect the changes in the claims as discussed in the telephone interview between Examiners Mear and Prouty and the Applicants' undersigned attorney. The interview took place on 16 January 2008. Applicants and their undersigned attorney wish to thank Examiners Mear and Prouty for granting the telephone interview and for providing helpful suggestions for amending the claims to remove indefinite expressions and to better distinguish over the cited prior art.

Antecedent basis for the amendments to claims 1, 14 and 26 and for new claim 28 may be found in the specification on page 5, line 14 through page 6, line 21; and in Example 1 on pages 23 through 26. Thus claims 1 through 8, 14 through 20, and 26 through 28 are now in the application and are presented for examination.

Examiner Prouty indicated that she believed that the claims last presented were much clearer than the claims as originally presented, but that she still wanted additional changes in independent claims 1, 14 and 26. Examiner Prouty indicated that the amended claims 1, 14 and 26, now presented, were more in line with the wording that she believed was appropriate to clearly define what the Applicants regarded as their invention. The independent claim 1 now presented make it clear that the recombinant nucleic acids according to the present invention

include at least one serine biosynthesis gene and a nucleotide sequence encoding L-serine dehydratase, which is partially deleted or is mutated, or fragments of the nucleotide sequence encoding L-serine dehydratase, flanking the 5' and the 3' end of said nucleotide sequence, encoding L-serine dehydratase to permit complete removal of said nucleotide sequence, including L-serine dehydratase by homologous recombination, and which is expressed to a lesser degree than the expression of the naturally occurring SEQ ID NO: 1 or is not expressed at all. Claims 14 and 26 have been amended to make it clear that the endogenous nucleotide sequence encoding L-serine dehydratase no longer encodes a protein with L-serine dehydratase activity.

Examiner Prouty questioned whether the Applicants are the first to isolate the L-serine dehydratase gene from Corynebacterium glutamicum and cited a Gen Bank Reference from NAKAGAWA published 5 June 2002. Examiner Meah sent the undersigned attorneys a copy of this reference by telefax and Applicants now enclose a copy of NAKAGAWA et al to complete te record.

Examiner Prouty indicated that NAKAGAWA is citable prior art, either per se or in combination with KUBOTA and LOVINGER et al, already of record. Thus Examiner Prouty indicated that NAKAGAWA may be anticipatory of claim 1 and perhaps NAKAGAWA in combination with KUBOTA and/or LOVINGER et al provides a strong argument for the obviousness of all claims.

Applicants agree only that NAKAGAWA was the first to publish the serine dehydratase sequence of Corynebacterium glutamicum. This is as assumed identical to serine deaminase, a term used also for the same enzyme. However, NAKAGAWA did not isolate the serine dehydratase gene. NAKAGAWA determined the genome sequence of C. glutamicum, and among the 3009 genes found, NAKAGAWA named one of the genes serine deaminase. NAKAGAWA neither cloned that gene, nor identified its function in an enzyme assay, nor used its sequence to derive an L-serine producer. Rather NAKAGAWA's denomination as a L-serine dehydratase (or deaminase) was only a result of comparison with a computer with other known sequences. Because of this there was no teaching about the function of the gene and the corresponding protein because real function can only be identified by experiments testing the activity of the enzyme encoded by the gene, as in an enzyme assay.

So regarding function in respect to the disclosure of NAKAWAGA, it could well be that the gene they inferred from the genome sequence could have a different function than deaminating L-serine, or it could even be that the gene was a pseudogene, that means fully inactive, without function.

Applicants do not know of any other disclosure in addition to NAKAWAGA which gives additional information about the function of the protein disclosed in new state of the art cited by the Examiner. But Applicants would like to emphasize that NAKAWAGA did not isolate the gene as assumed by the Examiner!

Thus Applicants were the first who identified the function of the gene and the correspondent protein. This means that Applicants were the first who found the main degradation path of L-serine.

KUBOTA disclosed an undirected mutagenesis to upgrade the formation of L-serine.

KUBOTA obtained a final concentration of L-serine of about 13.9 g/l. See the penultimate line of the abstract. LOVINGER did not disclose any data for L-serine formation so there is no teaching in respect to upgrade the L-serine formation by LOVINGER.

Contrary to anything disclosed in KUBOTA or LOVINGER, Applicants disclose in the present application that the L-serine dehydratase activity is unexpectedly, completely switched off when position 506 to 918 of the gene of L-serine dehydratase was deleted. This complete absence of L-serine dehydratase activity is disclosed in Table 2 page 31 of the specification in C. Glutamicum Strain 13032 ΔsdaA pXMJ19 transformed by such a form of L-serine dehydratase missing nucleotides 506 to 918. It is rather surprising that only a deletion of this part of the gene of L-serine dehydratase results in a complete deactivation of the protein. For this reason the subject matter of present claim 26 and especially claim 27 is especially believed to be surprising and unobvious, and therefore patentable over the prior art.

The complete deactivation of serine dehydratase in Applicants' approach led to a surprisingly high L-serine

accumulation of 35.7 gl. (340 mM) as published by Applicants in Applied and Environmental Microbiology 2007 (73) 750-755 (Fig. 4), by Stolz, Peters-Wendisch, Etterich, Gerharz, Faurie, Sahm, Fersterra and Eggeling, a copy of which is enclosed herewith. Should the Examiner so request Applicants are willing to make these data of record in a Declaration Under 37 CFR 1.132. Once again, KUBOTA, according to the abstract on the first page, obtained in his approach a final L-serine concentration of 13.9 g/I, a far lower concentration.

Since Applicants' deletion of only a part of the L-serine dehydratase gene according to present claims 26 and 27 results in a higher yield of L-serine than the L-serine yield as disclosed by KUBOTA using undirected mutagenesis, then one would reasonably expect that a complete deletion of the L-serine dehydratase gene will also result in a surprisingly better yield of L-serine than the L-serine yield as disclosed by KUBOTA. Thus the remaining claims, and especially new claim 28 are believed to be patentable over the cited prior art.

There is no correlation drawn between SEQ ID NO: 141 and SEQ ID NO: 142 disclosed in WO 01/00843, cited in the International Search Report and the International Preliminary Examination Report carried out by the European Patent Office in the corresponding European Phase of the instant PCT application, and the enzymatic activity of L-serine dehydratase. Copies of the both the International Search Report and the International Preliminary Examination Report have been made of record in the present

application. Furthermore, there is also no correlation drawn between SEQ ID NO: 141 and SEQ ID NO: 142 disclosed in WO 01/00843 to L-serine degradation.

In respect to patentability over the cited prior art, Applicants maintain the following:

KUBOTA is the closest prior art to be considered, because KUBOTA teaches that L-serine dehydratase catalyzes the degradation of L-serine. It is disclosed by KUBOTA in the abstract that the L-serine concentration can be increased to 13.9 g/l through mutagenic techniques. This is a result of an undirected mutagenesis of cells of Corynebacterium glycinophilum. Mutated cells of C. glycinophilum turned out to have somewhat reduced serine dehydratase activity. There is no teaching about a direct method to influence or even to exclude degradation of L-serine by L-serine dehydratase. Thus even after KUBOTA, the problem remains to find a method and substances which allow a complete prevention of L-serine degradation.

Applicants have solved this problem by preparing the recombinant nucleic acid according to claims 1and 26 and the claims dependent thereon, and a microorganism as claimed in claim 14 . In a special feature of the invention according to claim 27, a deletion of position 506 to 918 of the nucleic acid results in aa complete prevention of the L-serine degradation by L-serine dehydratase. In another special feature of the present invention, according to claim 28, a complete deletion of the polynucleotide

expressing L-serine dehydratase results also in a complete prevention of L-serine dehydratase activity.

In KUBOTA there is no disclosure or suggestion of how to completely eliminate the activity of L-serine dehydratase and how to completely prevent the degradation of L-serine. There is in fact no disclosure of the polynucleotide which is responsible for the degradation of L-serine.

LOVINGER does not disclose a quantitative result in which activity of L-serine dehydratase is decreased. There is also in LOVINGER no disclosure of the nucleic acids which are responsible for the degradation of L-serine. With both KUBOTA and LOVINGER in hand, one "skilled in the art" would be unable to develop strains and methodology to increase L-serine formation using Corynebacterium as the source of the L-serine biosynthesase by achieving a reduced degradation of the L-serine produced by completely eliminating degradation of the L-serine by the activity of L-serine dehydratase.

Neither KUBOTA nor LOVINGER nor the combination thereof discloses or suggests that the polynucleotide according to SEQ ID NO: 1 is responsible for expressing the L-serine dehydratase that catalyzes the degradation mechanism for degrading L-serine to pyruvate.

Thus the presently claimed invention is both novel and unobvious over the combination of KUBOTA and LOVINGER..

Regarding WO 01/00843 there is no disclosure of a function of the disclosed sequences. Indeed Table 1 of the

reference shows the sequences that express L-serine dehydratase. But this is just a result of sequence comparison via computer. The real function of a polynucleotide can only be identified by experiments testing the activity of the enzyme encoded thereby, as in an enzyme assay. WO 01/00843 discloses only the result of a genome sequence determination approach and no single gene was isolated or its activity assayed. Because of this, there is no teaching in WO 01/00843 about applicability of the disclosed sequences for reducing or eliminating L-serine dehydratase activity which is the result of experiences. Thus a deletion of a particular fragment of the L-serine deyhdratase gene, especially positions 506 to 918, or even the entire L-serine dehydratase gene, as presently claimed, is both novel and unobvious over any combination of KUBOTA, LOVINGER, WO 01/00843, and/or NAKAGAWA.

NAKAGAWA was the first to publish the serine dehydratase sequence of Corynebacterium glutamicum. This is as assumed identical to serine deaminase, a term used also for the same enzyme. However, NAKAGAWA did not isolate the serine dehydratase gene. NAKAGAWA determined the genome sequence of C. glutamicum, and among the 3009 genes found they found one which they termed serine deaminase. They have neither cloned that gene, nor identified its function in an enzyme assay, nor used its sequence to derive an L-serine producer. Rather the denomination as a L-serine dehydratase (or deaminase) was just a result of comparison with a computer with other known sequences. Because of this there was no teaching about the function of the gene and the corresponding

protein because real function can only be identified by experiments testing the activity of the enzyme encoded by the gene, as in an enzyme assay.

So regarding function in respect to the disclosure of NAKAWAGA, it could well be that the gene whose sequence they inferred from the genome sequence could have a different function than deaminating L-serine, or it could even be that the gene was a pseudogene gene, that means fully inactive, without function. This situation is completely analogous to the disclosure of WO 01/00843. So the person "skilled in the art would not be motivated to delete the gene encoding for "L-serine dehydratase" as disclosed by NAKAWAGA because the real function can only be identified by the experiments testing the activity of the enzyme encoded by the gene, as in an enzyme assay.

Because there is no teaching about the function of the gene disclosed by NAKAWAGA, there is an inventive step over the combination of KUBOTA, LOVINGER, NAKAWAGA and WO 01/00843. Once again, it was especially surprising that a deletion of only the nucleotides in position 506 to 918 of the L-serine dehydratase gene results in an complete inactivation of the L-serine according to claims 26 and 27.

Applicants believe that all claims now presented are allowable over the cited prior art and a response to that effect is earnestly solicited.

Respectfully submitted,
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Enclosures:
Applied and Environmental Microbiology, Feb. 2007, pp 750 to 755; and
BAB99038, Nakagawa, L-Serime Deaminase, 5 June 2002

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1: BAB99038. Reports L-serine deaminas...[gi:21324414]

BLink, Conserved Domains, Links

Comment Features Sequence

LOCUS BAB99038 449 aa linear BCT 05-JUN-20C2
DEFINITION L-serine deaminase [Corynebacterium glutamicum ATCC 13032].
ACCESSION BAB99038
VERSION BAB99038.1 GI:21324414
DBSOURCE accession AP005279.1
KEYWORDS .
SOURCE Corynebacterium glutamicum ATCC 13032
ORGANISM Corynebacterium glutamicum ATCC 13032
Bacteria; Firmicutes; Actinobacteria; Actinobacteridae;
Actinomycetales; Corynebacterineae; Corynebacteriaceae;
Corynebacterium.
REFERENCE 1
AUTHORS Nakagawa,S.
TITLE Complete genomic sequence of Corynebacterium glutamicum ATCC 13032
JOURNAL Unpublished
REFERENCE 2 (residues 1 to 449)
AUTHORS Nakagawa,S.
TITLE Direct Submission
JOURNAL Submitted (24-MAY-2002) Satoshi Nakagawa, Kyowa Hakko Kogyo Co.
Ltd., Tokyo Research Laboratories, 3-6-6, Asahi-machi, Machida,
Tokyo 194-8533, Japan (E-mail:snakagawa@xanagen.com,
Tel:81-44-829-3031, Fax:81-44-813-1651)
COMMENT This sequence is conducted by collaboration of Kyowa Hakko Kogyo
Co. Ltd. And Kitasato University.
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/db_xref="CDD:31946"
Region 176..439

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      121 ttrtylsvgg gfimtledfr kldqigsgvs tihpeaevpc pfqkssqlla ygrdfaevmk
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      241 ceigadldav ewvnlyslav neemaaggrv vtaptngaag iipavmhyar dflttgfgeq
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      421 grdmltkyke tslgglattl gfpvsmtcc
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APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 2007, p. 750–755 Vol. 73, No. 3
0099-2240/07/$08.00+0 doi:10.1128/AEM.02208-06


# Reduced Folate Supply as a Key to Enhanced L-Serine Production by *Corynebacterium glutamicum*▽

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**The amino acid L-serine is required for pharmaceutical purposes, and the availability of a sugar-based microbial process for its production is desirable. However, a number of intracellular utilization routes prevent overproduction of L-serine, with the essential serine hydroxymethyltransferase (SHMT) (*glyA*) probably occupying a key position. We found that constructs of *Corynebacterium glutamicum* strains where chromosomal *glyA* expression is dependent on $P_{tac}$ and *lacI*$^{Q}$ are unstable, acquiring mutations in *lacI*$^{Q}$, for instance. To overcome the inconvenient *glyA* expression control, we instead considered controlling SHMT activity by the availability of 5,6,7,8-tetrahydrofolate (THF). The *pabAB* and *pabC* genes of THF synthesis were identified and deleted in *C. glutamicum*, and the resulting strains were shown to require folate or 4-aminobenzoate for growth. Whereas the *C. glutamicum* Δ*sdaA* strain (p*serACB*) accumulates only traces of L-serine, with the *C. glutamicum* Δ*pabABC*Δ*sdaA* strain (p*serACB*), L-serine accumulation and growth responded in a dose-dependent manner to an external folate supply. At 0.1 mM folate, 81 mM L-serine accumulated. In a 20-liter controlled fed-batch culture, a 345 mM L-serine accumulation was achieved. Thus, an efficient and highly competitive process for microbial L-serine production is available.**

L-Serine is a nonessential amino acid but plays an important role in stabilizing the blood sugar concentration in the liver (16). It relates, furthermore, to many other substances, including sphingosine and the phosphatides, which are part of the myelin covering of the nerves, as well as the formation of activated $C_1$ units used for a number of anabolic processes (20). Therefore, L-serine is present in selected infusion solutions and also has other applications. For instance, it is an ingredient of skin lotions to ensure a proper hydration status. The total annual demand for L-serine is estimated to be 300 tons (5).

The production processes currently used still rely on the extraction of L-serine from protein hydrolysates or from molasses, as well as on the enzymological conversion of glycine plus a $C_1$ compound, like methanol, to L-serine. The latter uses the reverse reaction of the serine hydroxymethyltransferase (SHMT) (6). Thus, an enzymatic system has been designed to convert glycine plus formaldehyde to L-serine (4). The cellular systems assayed employed, among other things, resting cells of methanol-utilizing bacteria, such as *Hyphomicrobium methylovorum*, where L-serine formation from glycine plus methanol was achieved (6). In such a system, up to 45 g liter$^{-1}$ L-serine accumulation was possible, but only at a glycine yield of 50%, thus making the system less attractive. Also, alginate-entrapped cells of *Corynebacterium glycinophilum* were used for L-serine formation from glycine (21). It is self-evident that it would be most profitable to directly convert cheap sugar into L-serine. Although microbial processes for amino acid production are in general advancing quickly, attempts to develop L-serine producers have as yet yielded merely strains that form traces of this amino acid (7, 25).

We are engaged in exploring the production capabilities of *Corynebacterium glutamicum*, including flux directions, flux quantifications, and metabolite export, with the focus so far on L-lysine, L-isoleucine, L-valine, L-threonine, and D-pantothenate (2). Due to the apparent lack of a convincing strain for L-serine formation, we recently also explored the metabolism of this amino acid in *C. glutamicum*. We studied in detail the 3-phosphoglycerate dehydrogenase, SerA, catalyzing the initial reaction of the three-step pathway of L-serine biosynthesis (13). As a result of deletion of 197 amino acyl residues of the carboxy-terminal end of the SerA polypeptide, the 3-phosphoglycerate dehydrogenase activity is no longer inhibited by L-serine. Furthermore, we identified a high capacity of *C. glutamicum* to degrade L-serine, which is strongly reduced upon deletion of the *sdaA*-encoded L-serine dehydratase (11). Degradation is apparently a key issue in microbial L-serine formation, certainly with respect to the central role of this amino acid in metabolism. This agrees with the observation that overexpression of engineered *serA* together with *serB* and *serC* in *C. glutamicum* yielded only traces of L-serine (14). However, when the L-serine dehydratase gene was additionally deleted, a transient accumulation of up to 16 mM was observed. A further and substantial increase of up to 86 mM occurs when the SHMT activity is reduced, apparently by reducing L-serine degradation to glycine plus 5,10-methylene-tetrahydrofolate. Since the *glyA*-encoded SHMT is essential (14), a reduction of SHMT activity was required by a controllable promoter integrated in the chromosome. However, this strain is inconvenient, since it requires the control of iso-

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▽ Published ahead of print on 1 December 2006.

TABLE 1. Strains, plasmids, and oligonucleotides used in this study

| Strain, plasmid, or oligonucleotide | Relevant characteristics or sequence | Source, reference, or purpose |
|---|---|---|
| *C. glutamicum* strains | | |
| ATCC 13032 | WT[a] | Culture collection |
| 13032Δ*sdaA*::pK18mob*glyA*' (p*serACB*) | WT with *sdaA* deleted, regulatable *glyA*, and overexpression of *serA*(fbr), *serB*, *serC* | P. Peters-Wendisch |
| WTΔ*sdaA* | In-frame deletion of *sdaA* | P. Peters-Wendisch |
| WTΔ*sdaA*Δ*pabABC* | In-frame deletion of *sdaA* and *pabABC* | This work |
| WTΔ*sdaA*Δ*pabAB* | In-frame deletion of *sdaA* and *pabAB* | This work |
| WTΔ*sdaA*Δ*pabC* | In-frame deletion of *sdaA* and *pabC* | This work |
| WTΔ*sdaA*Δ*pabABC* (p*serACB*) | In-frame deletion of *sdaA*, and *pabABC*, overexpression of *serA*(fbr), *serB*, *serC* | This work |
| Plasmids | | |
| p*serACB* | pEC-T18mob2, Tet$^r$, containing *serA*(fbr), *serC*, and *serB* | P. Peters-Wendisch |
| pK19mob*sacB* | Km$^r$; mobilizable (*oriT*); *oriV* | A. Tauch |
| pK19mob*sacB*-*pabC* | Vector enabling deletion of 585 bp of *pabC* | This work |
| pK19mob*sacB*-*pabAB* | Vector enabling deletion of 1,734 bp of *pabAB* | This work |
| pK19mob*sacB*-*pabABC* | Vector enabling deletion of 2,475 bp of *pabABC* | This work |
| Oligonucleotides | | |
| pabAB-del-A | 5'-CGGGATCCTCAGGCTCGCACGTTGGAGGG-3' | Primer for 1,734-bp *pabAB* deletion |
| pabAB-del-B | 5'-CCCATCCACTAAACTTAAACAAAACGTGAAAGAATCATAATT-3' | Primer for 1,734-bp *pabAB* deletion |
| pabAB-del-C | 5'-TGTTTAAGTTTAGTGGATGGGGAGTGGGAGGAAATCCGCGTT-3' | Primer for 1,734-bp *pabAB* deletion |
| pabAB-del-D | 5'-GTGGATCCGCCCAAAACACCACGGTGGCGT-3' | Primer for 1,734-bp *pabAB* deletion |
| pabC-del-A | 5'-GAGGATCCAATCATTGCTGAGCTGCGCAG-3' | Primer for 585-bp *pabC* deletion |
| pabC-del-B | 5'-CCCATCCACTAAACTTAAACAATCAACAACTGTGGGTGTTGA-3' | Primer for 585-bp *pabC* deletion |
| pabC-del-C | 5'-TGTTTAAGTTTAGTGGATGGGTCGGTGAAGCCCTGGAATGAA-3' | Primer for 585-bp *pabC* deletion |
| pabC-del-D | 5'-AGGGATCCGTGATGAGTCCGATCTCGGAA-3' | Primer for 585-bp *pabC* deletion |
| pabABC-del-A | 5'-CGGGATCCTCAGGCTCGCACGTTGGAGGG-3' | Primer for 2,475-bp *pabABC* deletion |
| pabABC-del-B | 5'-CCCATCCACTAAACTTAAACAAAACGTGAAAGAATCATAATT-3' | Primer for 2,475-bp *pabABC* deletion |
| pabABC-del-C | 5'-TGTTTAAGTTTAGTGGATGGGTCGGTGAAGCCCTGGAATGAA-3' | Primer for 2,475-bp *pabABC* deletion |
| pabABC-del-D | 5'-AGGGATCCGTGATGAGTCCGATCTCGGAA-3' | Primer for 2,475-bp *pabABC* deletion |

[a] WT, wild type.

propyl-thio-β-D-galactopyranoside for production and tends to be unstable. We here describe our successful attempts to further improve the strain and to control the essential SHMT activity by a novel physiological approach.

## MATERIALS AND METHODS

**Bacteria, plasmids, and growth.** The bacterial strains and plasmids used in this work are listed in Table 1. Luria-Bertani medium (9) was used as the standard medium for *Escherichia coli*, while brain heart infusion medium (Difco) was used as complex medium for *C. glutamicum*. As minimal medium, CGXII was used (2), with 40 g liter$^{-1}$ glucose as a carbon source. When appropriate, growth of *C. glutamicum* strains was with kanamycin (25 μg ml$^{-1}$) or tetracycline (5 μg ml$^{-1}$). *E. coli* was grown at 37°C and *C. glutamicum* at 30°C in 50 ml to 100 ml medium in 500-ml baffled shake flasks with 120-rpm agitation with orbital shaking at a radius of 12.5 cm at 60% humidity. Growth of *C. glutamicum* 13032Δ*sdaA*::pK18mob*glyA*' was always in the presence of kanamycin. All production experiments were done at least twice with less then 10% variation.

**Construction of plasmids and strains.** Plasmids were constructed in *E. coli* DH5αMCR from PCR-generated fragments (Expand High Fidelity PCR kit; Roche Diagnostics) by using *C. glutamicum* ATCC 13032 DNA as a template. *E. coli* was transformed by the $RbCl_2$ method and *C. glutamicum* via electroporation (22). Homologous recombination and selection for gene deletion in *C. glutamicum* were done as described previously (18). All constructed plasmids were sequenced, and all transformants were analyzed by plasmid analysis and PCR with appropriate primers.

To enable *pabAB* deletion, pK19mob*sacB*-*pabAB* was constructed (8). For this purpose, primers pabAB-del-A and pabAB-del-B were used to amplify a 563-bp fragment of the 5' end of *pabAB* and primers pabAB-del-C and pabAB-del-D to amplify a 528-bp fragment of the 3' end of *pabAB*. The resulting PCR fragments were used in a second PCR with pabAB-del-A and pabAB-del-D as primers. The resulting 1,122-bp fragment was ligated into the BamHI restriction site of the mobilizable *E. coli* vector pK19mob*sacB*, leading to pK19mob*sacB*-*pabAB*. This was used to replace the intact chromosomal *pabAB* genes in *C. glutamicum* ATCC 13032 with the truncated *pabAB* genes, resulting in strain 13032Δ*pabAB*.

Similarly, pK19mob*sacB*-*pabC* was made for *pabC* deletion. In the first PCR, primers pabC-del-A and pabC-del-B were used with pabC-del-C and pabC-del-D, respectively. The resulting DNA was used in the second PCR with primers pabC-del-A and pabC-del-D, and the resulting 1,178-bp fragment was ligated into the BamHI site of pK19mob*sacB* to generate pK19mob*sacB*-*pabC*. This was used to generate 13032Δ*pabC*.

Plasmid pK19mob*sacB*-*pabABC* was made in a similar manner. In the first PCR, primers pabABC-del-A and pabABC-del-B were used with pabABC-del-C and pabABC-del-D, respectively. The amplification product was used in the second PCR with primers pabABC-del-A and pabABC-del-D, and the resulting 1,169-bp fragment was ligated into the BamHI site of pK19mob*sacB* to generate pK19mob*sacB*-*pabABC*. This was used to construct strain 13032Δ*pabABC*.

**Product formation.** For fed-batch fermentations, a 20-liter stirred-tank reactor (Bioengineering, Wald, Switzerland) was used. Cells were pregrown in shake

flasks in 160 ml CGXII up to an optical density of approximately 6 and used to inoculate the reactor, containing 8 liters of medium consisting of 0.2 g liter$^{-1}$ citric acid, 0.3 g liter$^{-1}$ $MgSO_4 \cdot 7H_2O$, 4.8 g liter$^{-1}$ $H_3PO_4$ (85%), 64 mg liter$^{-1}$ $MgSO_4 \cdot H_2O$, 40 mg liter$^{-1}$ $FeSO_4 \cdot 7H_2O$, 97 g liter$^{-1}$ corn steep liquor (African Products, Ltd., Sandown, South Africa), 15 g liter$^{-1}$ glucose, 15 g liter$^{-1}$ fructose, tetracycline (5 mg liter$^{-1}$), biotin (2 mg liter$^{-1}$), and 0.4 ml antifoam (Durapol 3000; Dow Plastics). The feed medium contained 350 g liter$^{-1}$ glucose plus 350 g liter$^{-1}$ fructose. For pH control, $NH_3$ (25%) and $H_3PO_4$ (1 M) were used. The feed started after the residual sugar concentration was <10 g liter$^{-1}$, which was adjusted to an amount which provided a constant relation between substrate and oxygen uptake. Temperature, pressure, pH, dissolved oxygen, consumption of antifoam, acid, and base, substrate mass, oxygen, mass of fermentation broth, and evolution of $CO_2$ were recorded online.

**Analytical methods.** Amino acids in the culture supernatant were determined by reversed-phase liquid chromatography after derivatization with *ortho*-phthaldialdehyde. Glucose and fructose were determined via reversed-phase liquid chromatography (Dionex, Sunnyvale, CA), and biomass was monitored by taking optical density measurements (600 nm) or by using a dry cell weight balance (Sartorius, Goettingen, Germany).

## RESULTS

**Stability of reduced *glyA* expression.** We previously found that for high L-serine accumulation, among other aspects, a reduced SHMT activity is necessary (14). This was achieved by placing in the chromosome the SHMT-encoding *glyA* gene under the control of $P_{tac}$, thus greatly reducing the SHMT activity if no isopropyl-thio-β-D-galactopyranoside is present. This leads to high L-serine formation while simultaneously reducing growth of the 13032Δ*sdaA*::pK18mob*glyA'* (p*serACB*) strain. We nevertheless found that of 17 fermentations on the 20-liter scale, only 4 displayed the expected high L-serine formation. In order to identify the reason for this apparent instability, the strain was cultivated in brain heart infusion medium on a 50-ml scale without isopropyl-thio-β-D-galactopyranoside and inoculated six times in series in the same medium, with each cultivation lasting 8 to 15 h. From the final culture, single colonies were derived and 10 of them analyzed by PCR with primer pairs amplifying the *glyA* locus as present in the wild type. Surprisingly, in one clone the wild-type situation was restored, indicating reorganization of the chromosomal *glyA* locus of the engineered strain. As a further means of characterizing the culture, from another 10 single colonies, primer pairs amplifying the *glyA* locus as present in the engineered strain were used to derive sequences of a 975-bp fragment encompassing $P_{tac}$ and parts of the repressor $LacI^q$. In three clones, the identical transition of C to T was detected, resulting in the exchange of Ala in position 13 of the $LacI^q$ repressor for Thr. In one further clone, T was mutated to C in sequences upstream of $lacI^q$. For a further confirmation in two clones with mutated $LacI^q$, the SHMT activity was determined. It was 48 and 40 nmol min$^{-1}$ mg (protein)$^{-1}$, respectively, instead of 8 nmol min$^{-1}$ mg (protein)$^{-1}$ determined for the control. Altogether this shows that the strain with SHMT activity controlled by $P_{tac}$ is explicitly prone to chromosomal mutations and rearrangements, thus making the strain unsuitable for large-scale fermentations.

**Analysis of the *glyA* locus.** Requiring a more stable and convenient strain, we searched for an alternative for controlling SHMT activity. Since SHMT activity requires pyridoxal 5'-phosphate as well as 5,6,7,8-tetrahydrofolate (Fig. 1) to catalyze L-serine conversion to 5,10-methylene tetrahydrofolate and glycine, we considered controlling SHMT activity with a limited supply of cells with 5,6,7,8-tetrahydrofolate. A genome

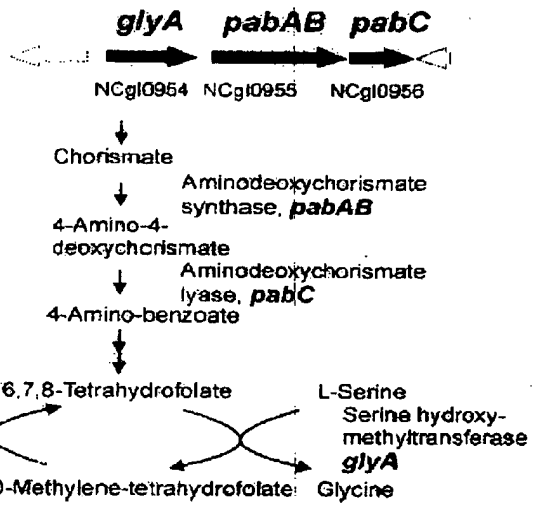


FIG. 1. Synthesis of tetrahydrofolate and its linkage to serine hydroxymethyltransferase. At the top is shown the genomic region of *C. glutamicum* encompassing nucleotides 1051865 to 1056075 of NC_006958, including *glyA*, *pabAB*, and *pabC*. Below are shown the corresponding enzymatic steps of tetrahydrofolate synthesis (simplified) and methylene tetrahydrofolate synthesis.

analysis revealed two open reading frames at the 3' end of *glyA* (NCgl0954) and transcribed in the same direction, putatively involved in tetrahydrofolate synthesis (Fig. 1). The N-terminal part of NCgl0955 shows strong sequence similarities (43% identity) to the *para*-aminobenzoate synthase component I (PabA) of *E. coli*, whereas its C-terminal part resembles the *para*-aminobenzoate synthase component II (PabB; 38% identity). Apparently, in *C. glutamicum* both polypeptides involved in the synthesis of *para*-aminobenzoate are fused, which is also the case for *Corynebacterium efficiens* and *Corynebacterium diphtheriae* (not shown) but not in the related species *Mycobacterium tuberculosis* and *Mycobacterium bovis*. The product of the PabAB activity is 4-amino-4-deoxychorismate, and NCgl0956 might encode the lyase, PabC, subsequently converting this product within the tetrahydrofolate pathway into *para*-aminobenzoate and pyruvate (3).

**Construction and analysis of folate auxotrophs.** Using the appropriate allelic-exchange vectors, the genes *pabAB*, *pabC*, and *pabABC*, respectively, were deleted from the chromosome of 13032Δ*sdaA* (see Materials and Methods). The resulting strains were streaked on minimal medium CGXII without further additions to assay for their folate auxotrophy. However, there was no visible difference in growth from that of wild-type cells. After a subsequent transfer onto a further minimal medium plate, colonies were somewhat smaller than the control, but only after a third transfer was no growth of strain 13032Δ*sdaA*Δ*pabABC* and 13032Δ*sdaA*Δ*pabAB* apparent, whereas there was still limited growth of 13032Δ*sdaA*Δ*pabC* (Fig. 2). The growth of all three mutants could be fully restored by supplementing either 1 mM folate or 4-aminobenzoate. The partial growth of

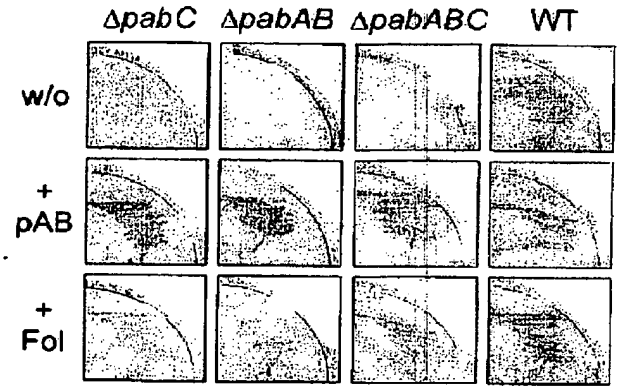


FIG. 2. Growth of mutants of *C. glutamicum* deleted of genes of folate biosynthesis. Growth of the corresponding mutants compared to that of the control (WT) without vitamin addition (w/o), plus 1 mM 4-aminobenzoate (+pAB), or plus 1 mM folate (+Fol). All strains were isogenic with respect to *sdaA* deletion.

the Δ*pabC* mutant could indicate that the substrate of the *pabC*-encoded enzyme is also nonenzymatically converted in *C. glutamicum*, which is in accord with results reported by Tewari et al. (23) showing that the intermediate 4-amino-4-deoxychorismate is labile and decomposes spontaneously to 4-aminobenzoate.

**Growth and L-serine accumulation by 13032Δ*sdaA*Δ*pabABC* (p*serACB*).** Based on the observation that the *pabABC* deletion was more favorable than that of *pabC*, *C. glutamicum* 13032Δ*sdaA*Δ*pabABC* was transformed to tetracycline resistance with p*serACB* to determine its L-serine production capabilities. The resulting strain was cultivated overnight in complex brain heart infusion medium and subsequently transferred to minimal medium CGXII without any addition of folate. After growing for 10 h, cells of this culture were used to inoculate the main culture (CGXII) with different folate concentrations. The resulting growth curves are shown in Fig. 3 (top). Without the addition of folate and with the lowest folate concentration of 0.01 mM, growth of strain 13032Δ*sdaA*Δ*pabABC* (p*serACB*) was severely impaired and the growth rate did not exceed 0.1 $h^{-1}$. The weak growth without the addition of folate was due to traces of folate still present in the inoculum, since cells taken at the end of the cultivation to inoculate a new culture did not grow. The addition of 1 mM folate fully restored growth of the auxotrophic strain so that it was almost identical to that of its ancestor strain, 13032Δ*sdaA* (p*serACB*), and the intermediate concentrations of 0.1 and 0.25 mM enabled partial growth with respect to both rate and final cellular optical density reached.

Whereas with the control strain 13032Δ*sdaA* (p*serACB*), the L-serine concentration was in the micromolar range, with 13032Δ*sdaA*Δ*pabABC* (p*serACB*) and 1 mM folate, up to 1.8 mM L-serine accumulated (Fig. 3, bottom). Lowering the folate concentration to 0.25 mM drastically increased the L-serine accumulation to concentrations of up to 60 mM. Even further-increased L-serine concentrations, up to 94 mM, were

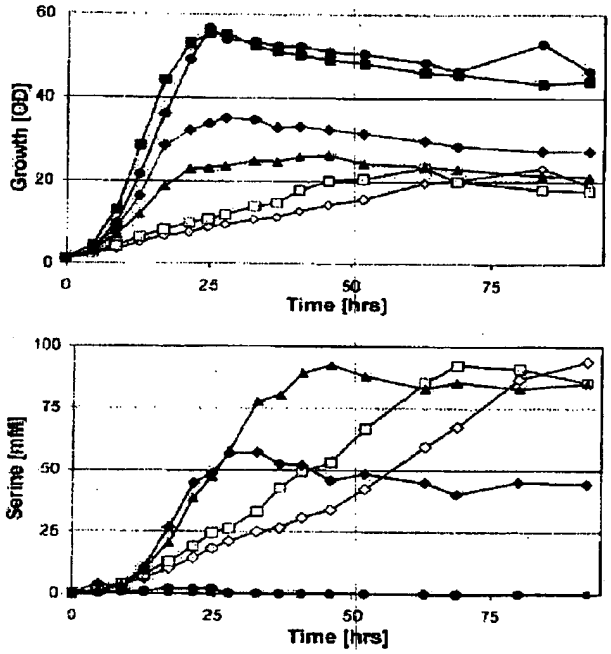


FIG. 3. Growth (top) and L-serine production (bottom) of *C. glutamicum* 13032Δ*sdaA*Δ*pabABC* (p*serACB*) in minimal medium containing different folate concentrations (◇, 0 mM; □, 0.01 mM; ▲, 0.1 mM; ◆, 0.25 mM; ●, 1 mM). The control strain, 13032Δ*sdaA* (p*serACB*), did not receive folate (■). OD, optical density.

obtained upon reducing the folate concentration to 0.1 mM. High final L-serine titers were also obtained at a concentration of 0.01 mM and without folate addition, although this required extended production times.

**L-Serine accumulation on an increased scale.** As is evident, reduced folate availability is promising for assaying for L-serine formation on a larger scale. In order to also investigate the properties of the strain constructed under such conditions and in a less-defined medium probably more relevant for industrial conditions, the performance of strain 13032Δ*sdaA*Δ*pabABC* (p*serACB*) was evaluated by using a 20-liter reactor based on corn steep liquor medium. The medium contained 35 g $liter^{-1}$ solid corn steep liquor plus initially 15 g $liter^{-1}$ glucose and 15 g $liter^{-1}$ fructose. The minimum dissolved oxygen concentration was set to 50% saturation to ensure no oxygen limitation. As can be seen in Fig. 4, inoculation of the reactor with cells derived from the preculture CGXII enabled rapid growth, up to a maximum specific growth rate of 0.25 $h^{-1}$. L-Serine formation occurred from the beginning up to a final concentration of 345 mM, suggesting a suitable folate supply in the culture due to corn steep liquor use, which can be assumed to contain at least traces of folate. The maximum oxygen uptake rate was about 110 mol $liter^{-1}$ $h^{-1}$, which was present at the end of the logarithmic growth of the culture. The maximal specific productivity was 1.45 mmol $g^{-1}$ $h^{-1}$, and the volumetric productivity was about 1.4 g $liter^{-1}$ $h^{-1}$.

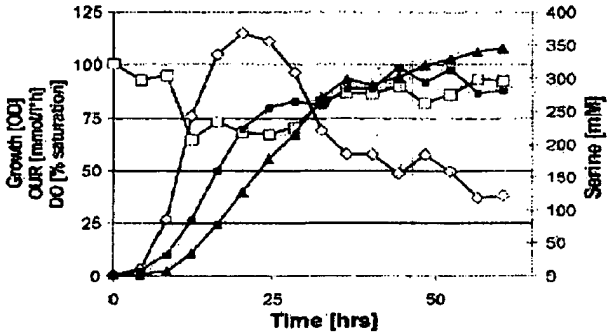


FIG. 4. Performance of *C. glutamicum* 13032Δ*sdaA*Δ*pabABC* (p*serACB*) in a 20-liter reactor showing growth (■), the accumulation of L-serine (▲), the dissolved oxygen (DO) saturation (□), and the oxygen uptake rate (OUR) (◇). OD, optical density.

We did not observe a stability problem with this strain, since two further fermentations gave reliable high L-serine titers with a variation below 10%.

## DISCUSSION

The SHMT is essential in *C. glutamicum*, as is also the case for other organisms (14). Besides glycine, the enzyme activity generates the activated one-carbon units required for a number of cellular processes, for instance, for the synthesis of formylated methionine bound to the initiator $tRNA^{fMet}$, which is necessary for translation initiation. This cellular demand cannot be bypassed by external metabolite addition. Furthermore, SHMT activity is involved in the generation of a number of metabolites and reactions, and it is therefore not surprising that the total carbon flux towards L-serine on minimal medium with glucose as the substrate amounts to 7.5%, as estimated for *C. glutamicum* (10). Earlier estimates for *E. coli* determined that as much as 15% of the carbon assimilated from glucose involves L-serine (15). Due the high demand and its key position in cellular physiology, L-serine has to be regarded as an intermediate of the central metabolism (20). These aspects together might explain the strong selective pressure against possession of a genetic construct with an adjustable *glyA* promoter, as we initially used in our studies (13, 14) and where mutations became apparent.

To overcome the stability problem, we generated a folate-auxotrophic strain. Although folate is essential, as is SHMT activity, the strain can be cultivated without change due to the external supply of this vitamin and the absence of identical chromosomal sequences available for recombination. The folate requirement was visible only after starvation for the vitamin by precultivation without adding the vitamin. We similarly observed this for pantothenate-auxotrophic strains of *C. glutamicum* (11), and it is obviously due to the requirement for vitamins in very low and catalytic concentrations only. This also suggests a low biosynthetic capacity for vitamin synthesis compared to the synthesis of a central metabolic building block. Thus, folate and pantothenate biosynthesis genes are assumed to be expressed at a low level compared to *glyA*, the SHMT gene. Indeed, enzyme activities point towards this direction, since in *C. glutamicum* the pantothenate biosynthesis enzymes have specific activities below 1 nmol $min^{-1}$ mg $(protein)^{-1}$ (17), and for *p*-aminodeoxychorismate synthase in *E. coli*, a comparably low specific activity was also determined (24). In contrast, SHMT activity in *C. glutamicum* is about 40 nmol $min^{-1}$ mg $(protein)^{-1}$ (14), and the protein is easily detectable in two-dimensional gels (19). Interestingly, a Northern analysis revealed a strong monocistronic message of *glyA* (data not shown), whereas we were unable to detect a message for *pabABC*. Since *glyA* is separated by just 75 bp from *pabAB* (Fig. 1) and no rho-independent terminator is apparent between both genes, this suggests an interesting expression control of the cluster.

Two L-serine conversion reactions are recognized in *C. glutamicum* whose cellular reduction is the key to achieving L-serine accumulation. As our previous studies have shown, SHMT activity clearly has a major impact (11, 13, 14). The reduction of *glyA* expression alone resulted in an approximately 1 mM accumulation of L-serine (13), which was not the case upon deletion of the serine dehydratase gene *sdaA*. As the present work has shown, limitation of folate is an ideal tool for limiting L-serine conversion and directing its flux towards extracellular L-serine. Similarly, control of D-pantothenate availability is known to influence the formation of selected amino acids. The basis is that D-pantothenate is a constituent of coenzyme A, and a reduced coenzyme A availability results in reduced activity of the pyruvate dehydrogenase, thus limiting pyruvate decarboxylation. This has been exhaustively used in developing a *C. glutamicum* strain producing L-valine, which is made up of two pyruvate molecules (17). It should be noted that vitamin limitations in strain constructions are entirely different from the well-established "pathway tailoring" by removing competing reactions or removing bottlenecks (1). The reason is that at a fixed low vitamin concentration, the cell as a catalyst is still active but its proliferation reduced, which might affect in many ways the physiology of the cell. For instance, in *E. coli* a YgfZ protein is present, which may be a folate-dependent regulatory protein involved in C-1 metabolism (12), and a similar protein is present in *C. glutamicum* (NCgl2492).

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